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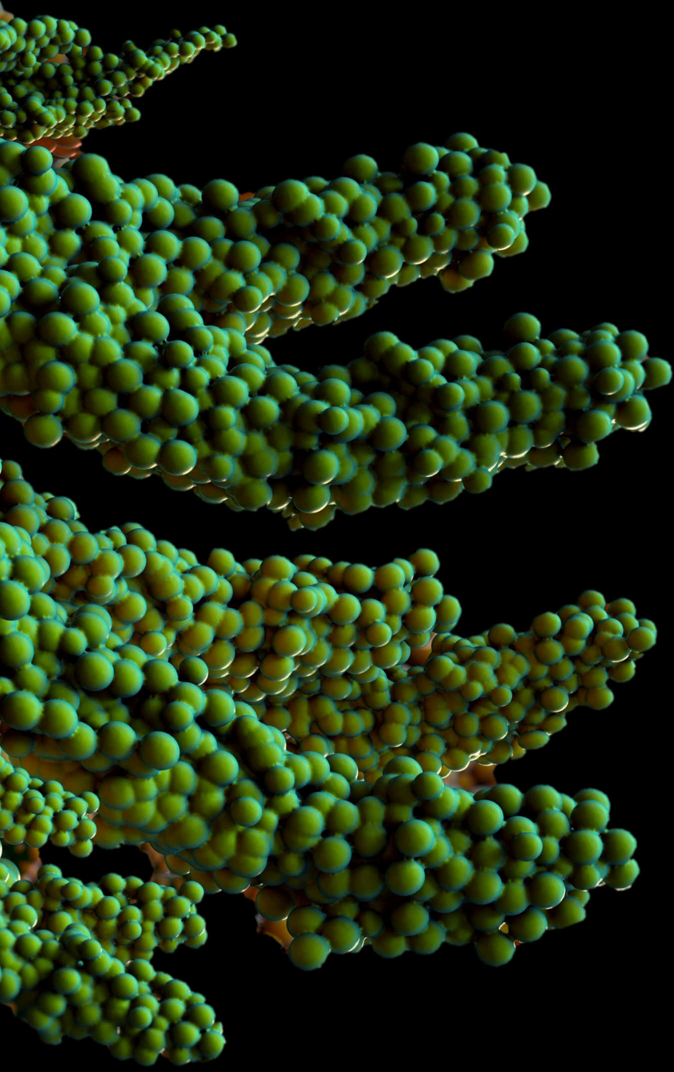

TOPICAL:

Mitigation and Use of Biofilms in Space for the Benefit of Human Space Exploration

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Abstract
Biofilms are groups of cells of the same or different species living in communities. Such structures are usually attached to surfaces, and on Earth, they have been found in diverse and extreme environments. Such agglomerations have been described as recalcitrant or protective when facing adversity. In space systems, biofilms have been found on a multitude of hardware surfaces. Different studies have identified genetic changes that may impact human health. The insufficiency of consistent research may return inconclusive arguments as to what extent microgravity encourages virulence and how biofilms could exacerbate crewed spaceflight – especially ones to remote areas with a lack of resupply and service missions. However, biofilms, are also beneficial to plant biology, and they may supply in metabolic pathways that produce useful organic and inorganic components to maintain life in other celestial bodies. There are expansive areas of research that look into biofilms in space and scientific recommendations that reflect on expanding the aerospace industry's knowledge of biofilms, how to mitigate, or use them to the advantage of spaceflight.

Introduction: *Current Issues in Spaceflight*

Biofilms are multicellular communities of microorganisms embedded in an extracellular polymeric matrix that may be found attached to surfaces or floating in a liquid[1,2]. A mature biofilm is the result of a multistep process of surface attachment, maturation and detachment[3]. Its structure allows for cells to live inside a "protective layer" that influences virulence[4] and causes heightened antimicrobial resistance[5]. Different microbial biofilms can result in resistance to other extreme environments, such as UV, extreme pH levels, high or low temperatures, nutrient starvation, high salinity, and pressurized environments[6]. When on surfaces, biofilms are known to affect hardware materials leading to potential failures in a variety of industrial and clinical systems[7,8]. Sewage systems on Earth are known to be contaminated by biofilms that can transport antimicrobial resistance genes that may affect water and medical treatments[9,10]. However, biofilms can be beneficial when developing microbial fuel cells[11], in bioremediation[12], and certain food product bioprocesses[13]. Biofilm has been found on a multitude of surfaces in previous space missions. These surfaces being: the water recycling systems, hatch locks, control panels, electrical connectors, oxygen electrolysis block, thermal control system's radiator, EVA suit's headphone, and navigation window[14,15]. The components above are of high importance when it comes to supporting life in space by directly supplying the basic necessities of life or mission controls. New missions may require similar hardware to the previously mentioned, necessary to support crewed operations, as existing systems supply "lessons learned"[16]. Due to the risk that biofilms represent, it is relevant to continue studies aimed to understand their cycle and physiology, their effects on surfaces, and to develop potential mitigation practices. Pathogenesis, on the other hand, has a direct effect on astronaut health, and the effects of biofilms in differing gravities are only partially understood due to the limitations of microgravitational studies and the complexity of working with different species of biofilms and describing their similarities across the board[17,18]. In contrast, optimizing biofilm genomes for production (e.g., for nutrient production/extraction and plant microbiology) may enable more autonomous space exploration[19,20,21]. This decade's goals for human exploration of the Moon and beyond serve as catalyst for interest in microbial studies relevant to space environments (*see graphic diagram* **Biofilm - Crewed Missions**).

Wet Surfaces

Spaceflight hardware used during missions may have surfaces in direct contact with liquids; whether these are fuels or crew water for different uses, they are at risk for microbial contamination and propagation. Although biofilm contamination has been observed in aircraft fuel tanks[22], contamination of propellants has been studied, when appropriate, by the National Aeronautics and Space Administration (NASA) as part of their Planetary Protection risk mitigation activities[23]. However, fuel tanks recycled for use in other celestial bodies may have not been tested for biofilm hardiness in faraway space environments, such as the Moon and Mars. In other industries, materials such as stainless steel and aluminum alloys (common fuel tank materials) have been studied for corrosion susceptibility[24,25], but these conditions are not well understood in microgravity. A constant wet surface considered in crewed missions is that related to the water recycling systems. This system can comprise the water processor assembly (WPA) to produce drinking water, potable

Biofilm - Crewed Missions

National Aeronautics and Space Administration

Russian condensate processor biofilm. Image provided by NASA.

A - Environmental Control and Life Support Systems
B - Air recirculation Vents
C - First Aid Kit
D - Showers
E - Toilet
F - Dry Surfaces
G - External Resources
H - Food
I - Miscellaneous Tubing
J - Plants

Mold on International Space Station (ISS) interior wall. Image provided by NASA.

water dispenser, and any necessary cleaning and research water (See images A, B, D, E, I, and J). In recent times, parts related to the WPA have been grounded due to biofilm obstructions[26]. In the case of future space missions, systems must resist or avoid a constant influx of microorganisms, especially those that cannot be serviced due to mission distances. Multiple efforts have been made to mitigate and treat biofilms in WPA systems[27,28,29]. However, space systems studies have been noticeably affected by the crew's microbiome and nutrient availability[30,31]. Biofilm-related studies have not just given light on the aforementioned topics but also pointed to knowledge gaps in the way biofilms are tested[32], the role of microgravity in microbial behavioral differences, and how different species affect the outcome of such results[33].

Astronaut Health

Medical care in ISS is restricted by payload capacity of flights[34], and ability to service the crew, and thus treatment of astronauts can become more difficult the further away from Earth the modules, habitats, or stations are (Example of a first aid kit used in space, image C). On Earth, medical devices are studied for their capacity to resist biofilms (bacterial and fungal), due to the danger it represents to patients[35], which is applicable to in-space habitation. Studies have blamed the space environment for increased virulence[36], although contradicting results have created doubts on the actual effects of microgravity[37], while others pointed to biofilm formation, species, and growth conditions as factors of increased virulence in microgravity[38]. As the studies listed in *Table 1* may not be universally applicable to different microorganisms, follow up studies must be performed before humanity may delve into more distant celestial bodies.

	Table 1. Results from health-related studies have concluded, among a few others, that:
1	NASA has identified increased immune dysregulation as a threat to crew safety and mission success[39,40];
2	International Space Station (ISS) microbial isolates have shown the contribution of individual community members to the robustness of polymicrobial biofilm formation[41];
3	polymicrobial evolution occurs, there are changes in microbial interactions between co-habiting species over time, and there is microbial long-term adaptation and evolution within communities[42-46];
4	microbial genomes in station have been described and some isolates clearly show presence of drug-resistance genes[47], such as on Earth;
5	plasmids are present just as expected in this closed environment (thus the ability to carry drug resistance)[48];
6	but resistance spread and biofilm virulence factors in single species and polymicrobial biofilms are not well understood in microgravity

A special topic referred to in the aspect of astronaut health and wet surfaces is the use of biofilm detection systems. Mechanical biofilm detection systems are based on microgravimetry and measure the amount of biofilm mass formed on piezoelectric films. Several other techniques have been employed in biofilm monitoring, including the use of quartz crystal microbalances[49], quartz tuning forks[50], and surface acoustic wave sensors[51]. These sensors are low cost and offer real time data; however, their integration into spaceflight systems is challenging due to required crew interaction and low vibrational tolerance. Within the constraints of spaceflight, electrochemical impedance spectroscopy sensors offer the greatest promise, and have been the most commonly used for biofilm detection[52]. Impedance sensors can be microfabricated using matured semiconductor technologies resulting in precise manufacturing of small (~ nm to µm feature sized), lightweight, highly sensitive, minimally invasive sensors that require low power (~<50 mW) to operate. Prior to deployment of impedance-based biofilm sensors in critical water recovery and life-support systems several challenges have yet to be addressed. It is not known how these systems function in micro-gravity; however, because electrochemical impedance relies on biofilm surface interactions it is believed that this will not be an issue. Proposed timescales for spaceflight operations vary from a few days to years and extended periods of dormancy may also occur in landers, gateway stations, or spacecraft. In future studies sensor robustness needs to be characterized or alternative strategies requiring sensor exchange need to be developed.

Food and Plant Research

Bacteria associate with roots of plants as biofilms, and pathogenic bacteria often have an enhanced biofilm forming capacity[53]. The formation of food pathogen biofilms can have serious health consequences, an example of which occurred in the US in 2016 where Shiga toxin producing Escherichia coli infected alfalfa sprouts[4]. The analysis of plant-microbe interaction has demonstrated and underscored the importance of microorganisms and their biochemistry in the healthy functioning of terrestrial plants[55]. For example, the nitrogen-fixing rhizobacteria, that attach to plant roots and form biofilms, are important players in a healthy plant-microbe relationship. To date 12 Veggie space crop experiments have been conducted on the International Space Station of these, so far, the VEG-01A, VEG-01B, and VEG-03A "Outredgeous" Red Romaine lettuce samples were evaluated by microbiome analysis using Next Generation Sequencing on the Illumina MiSeq[56] platform. Here, robust microbial communities were observed along with no human pathogens. The microbiological counts on spaceflight-grown produce during VEG-01A, VEG-01B, and VEG-03A were determined to be no more abundant than store-bought produce. This baseline survey begins to build our understanding of the microbiome associated with space crops.

Food safety. Prior to being consumed by the crew, Veggie crops are sanitized with PRO-SAN® wipes from MICROCIDE®, a citrate-based treatment. Further investigation into alternative options such as UV, ozone, and plasma are underway.
Knowledge Gaps. Johnson Space Center conducts food safety studies to ensure Veggie crops are safe to consume. Kennedy Space Center conducts space crop production studies to understand the environmental conditions which shape the plant microbiome interactions. Gaps in this knowledge include how to best water crops and how to control humidity in the low convection of the spaceflight environment to avoid the growth of opportunistic pathogens (including fungi).
Recommended Studies. Continue plant stress surveillance and experimentation in controlled ground-based simulated spaceflight environment studies and space flight studies through advanced imaging, sequencing, and metabolomics to allow for the identification of key beneficial microbes and pathogens and their associated genotypic and phenotypic outcomes. This will allow for a level of prediction and ecosystem control for the spaceflight environment.

Figure 1. Veggie sanitation, knowledge gaps, and recommended studies.

Dry Surfaces

As biofilms thrive in continuously wet or moist conditions, dry surfaces tend to represent an unfavorable environment for biofilm formation. However, dry hard nonporous surfaces on the ISS can be intermittently damp due to fluctuating humidity levels, and experience high touch contact during normal usage[57]. In general, the ISS microbial community has been shown to consist of human-associated microbes, which can be transient or enduring, and consist of various types of bacteria and fungi[58-63]. It is critical that we understand the means by which microbes become deposited on surfaces, which is likely through means of direct contact and air circulation since aerosol settling is not a factor in microgravity[64]. Others are asking how important the surface material is in the development and morphology of the dried biofilm[65,66,61]. Possible threats of biofilm growth have been identified and include microbially induced corrosion (MIC) and blockage of mechanical components. The ISS makes use of humidity and condensation controls[57,67,68], HEPA filters, cleanable surfaces[57], as well as cleaning of materials sent to the ISS[60]. Furthermore, human microbial communities are monitored both pre- and during flight[69-71]. Last, measures are taken to frequently clean the ISS using a variety of methods[72]. In short, these cleaning practices include weekly or daily cleaning with disinfecting wipes and vacuuming for larger debris[73]. In the context of a biofilm, it is not always easy to distinguish between surface cleaning (i.e. removing the dried biofilm) and sanitization (i.e. killing or inactivating cells). Some chemistries and procedures kill viable cells but do not remove the matrix (e.g. quaternary substances, most antimicrobial surfaces), some remove the matrix but do not kill the cells (e.g. scrubbing with a microfiber cloth), some do both (e.g. bleach). Given that wipes are frequently employed on

Table 2. Dry Surfaces: Knowledge Gap Questions
1.How does the unique environment on the ISS impact the development of dried biofilm? Does less or more biofilm form on the surfaces of the ISS? Enhanced survival mechanism? How does microgravity effect the matrix, and does this matrix respond to antimicrobial surfaces in same as on Earth?
2.How do surface communities change over time, for instance community structure and composition, lateral gene transfer, and mutations. Research is necessary to continue the studies in progress.
3.How durable are antimicrobial surfaces? Is the durability shorter or longer than on Earth?
4.What biosurveillance/sensor tools are available to monitor surface growth?
5.Could we monitor another parameter (temperature/metabolic activity) rather than look for the microbes themselves?
6.How do multigenerational changes in microbial physiology & genetics in partial gravity, enhanced radiation and possibly altered chemical environment beyond low Earth orbit (LEO) impact biofilm growth? Do these changes impact our ability to kill, remove and/or control biofilm growth?
7.On-board sterilization protocols in the event that some surgical procedure may be needed (e.g., fractures, appendectomy, etc.) – crew recovery following trauma unknown – would be a significant medical issue for future mission beyond LEO, surfaces for surgery, surfaces of implants or of tools (Knowledge for life well beyond LEO)
8.Microbial technology (sequencing, computing, detection, 3-D printing of materials (and recycling of such materials) etc.) to reduce dependency on Earth support. Potential for recycling/regeneration of materials that can be used (cloth, disinfectants, etc.) again to reduce Earth dependency.
9.As humanity aims to return to the moon, and eventually travel to Mars, it is inevitable that microbes will be delivered to these surfaces. Thus, microbial associations and interactions with external materials (regolith, rocks, and minerals) that are not terrestrial should also be explored to understand their effects on microbial growth. Furthermore, what are the influences of microgravity and partial gravity (i.e. that of the moon or mars) along with radiation beyond low Earth orbit on microbial growth[77-82].

the ISS, it is expected that this provides mechanical removal of the dried biofilm, and thus is beneficial in achieving both aims. Finally, precaution must always be taken to consider toxicity of compounds used for cleaning— particularly volatiles—within a closed system like the ISS[63]. While antimicrobial surfaces have been extensively explored as an approach to control microbial contamination on hard non-porous high touch surfaces in a hospital environment[74], the use of antimicrobial surfaces has not yet been implemented on the ISS[63]. However, studies by the European Space Agency (ESA) and Boeing are ongoing now aboard the ISS to assess antimicrobial nature of materials for use in future applications[75,76].

In-Situ Resource Utilization (ISRU)

Although biofilm formation is often seen as a negative occurrence, biofilms could potentially play a positive role in space travel through their use in various ISRU procedures. Biomining is the blanket term for the processes by which a biological system – typically a bacterial biofilm – extracts and recovers desired metals from rock ores. This process can be divided into the more specific methods of bioleaching and bio-oxidation and is currently in use on Earth[83]. When it comes to extracting useful metals in space, biomining is more advantageous than traditional mining methods as it is a lower energy process, is less toxic, and takes up much less equipment area[84]. Bioleaching (removing the target compound by dissolving it via redox biochemistry) has been studied with extraterrestrial purposes in mind[85]. The ESA has conducted BioRock biomining studies aboard the ISS and found that multiple gravitational conditions did not prevent the effective bioleaching of vanadium (an element of interest due to its strength and resistance to corrosion) from basalt rock[86]. Two of the bacterial strains used increased vanadium leaching, one by up to 283.22%[87]. Data from the BioRock project shows that biomining "may be possible on a large scale in space", enabling extraction of elements necessary to human survival outside of Earth[84]. Aside from biomining, biofilms can be utilized in space exploration and ISRU through bioregenerative life-support systems. The toxic dust of Mars presents many issues when thinking of manned missions. A potential solution for controlling this dust may be found in Cyanobacterial biofilms. In an area of Mars-like Mongolian desert, sand was seeded with cyanobacteria which within a timeframe of 15 days produced stable, wind-resistant crusts that prevented the release of dust particles[88]. Racks of these crusts could serve an air filtration purpose by removing dust from the atmosphere as it passes through. Microbial crusts and biofilms could also aid in extraterrestrial plant growth and regolith-to-soil processes, aiding in both morale and survival for astronauts[89]. To expand upon the aforementioned uses - employment of microbes, often as biofilms, has been proposed for production and recovery of resources, such as generation of oxygen, food and materials, biomining, wastewater recycling, as well as generation of energy and even terraforming[90-95]. For extraterrestrial food production, photosynthetic bacteria, *Arthrospira platensis* and *Arthrospira maxima* (together forming the nutrient rich supplement Spirulina) have been identified as a competitive and complimentary option to plant-based space farming[96,97]. Production of medicines on Mars will be important to avoid degradation by radiation and temperature variations[98]. Many pharmaceutical molecules can be produced efficiently on-demand using compact microbial bioreactors[99-101]. *Pichia pastoris* could potentially be an ideal production host for medicines, metabolites, and materials on Mars, due to their extensive gene-engineering tools, and its metabolic versatility - it can grow on methanol, derived from methane, a possible ISRU product on Mars[90,102]. Biomining and bioremediation for extraction and recovery of rare Earth elements, precious metals, removal of perchlorate, are important strategies for bio-ISRU. Several microorganisms have been utilized in proof-of-concept experiments for biomining on Earth, as well as the proving-ground of the ISS[103-111]. In particular, *Acidithiobacillus ferroxidans, Cupriavidus metallidurans, Shewanella oneidensis* and *Sphingomonas desiccabilis* are promising, most of these species performing chemolithotrophic leaching. Due to various methods mentioned here, as well as many other potential applications, biofilms and microbes in general prove to have potential value in space ISRU.

Conclusion

NASA has a long history of utilizing research and lessons learned to improve methods, build upon current procedures, and produce models to achieve the best possible practices. Rigorous scientific study coupled with a continual willingness to examine and improve are critical to answer some important questions in biofilm research and to ultimately help provide the best chance of success in long duration space missions when it comes to biofilm mitigation and use of microbial sources to sustain life.

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